Brief Report

The direct PAK1 inhibitor, TAT-PAK18, blocks preferentially the growth of human ovarian cancer cell lines in which PAK1 is abnormally activated by autophosphorylation at Thr 423

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ABSTRACT: So far no effective therapeutic has been developed for the FDA-approved treatment of ovarian cancer patients. Recently we provided the first evidence indicating that an old antibiotic (anti-parasitic drug) called Ivermectin suppresses the growth of a variety of human ovarian cancer cell lines *in vitro* by inactivating the oncogenic kinase PAK1 somehow (Hashimoto H, *et al. Drug Discov Ther. 2009; 3:243-246*). This kinase is now known to be essential for the growth of more than 70% of all human cancers including breast, prostate, pancreatic, colon, gastric, lung, cervical, thyroid cancers as well as hepatoma, glioma, melanoma, MM (multiple myeloma) and NF (neurofibromatosis) tumors. In this study, using the cell-permeable PAK1-inactivating peptide TAT-PAK18 which blocks the essential PAK1-PIX interaction, we examined the relationship between the sensitivity of ovarian cancer cell lines to this anti-PAK1 peptide and the protein expression/autophosphorylation levels of PAK1 in these cell lines, and found that the more PAK1 is abnormally activated (autophosporylated at Thr 423), the more their growth is sensitive to this peptide, regardless of their PAK1 expression levels. This observation provides the first direct evidence that ovarian cancers also belong to the PAK1-dependent cancers which represent more than 70% of all human cancers, suggesting that anti-PAK1 drugs would be effective therapeutics for ovarian cancers.

Keywords: TAT-PAK18, PAK1, ovarian cancer, PIX, autophosphorylation

1. Introduction

Among female-specific cancers, breast, ovarian and cervical cancers are the major three that cause the death of many cancer patients world-wide. Around 75% of breast cancers require estrogen for their growth, and the remaining 25% appears to be estrogen-independent. Thus, in the past the former estrogen-dependent breast cancers have been treated by an estrogen antagonist called Tamoxifen (Tam) with a relatively great success. However, after a prolonged treatment, these breast cancers become Tam-resistant, mainly because the kinase PAK1 is abnormally activated (*1*). We have shown that the growth of these Tam-resistant breast cancers as well as estrogen-independent breast cancers can be almost completely suppressed by anti-PAK1 drugs such as FK228 and combination of AG 879/GL-2003 and PP1 (*2,3*) *in vivo* (human cancer xenografts in mice). Similarly, the growth of human ovarian cancer cell lines can be suppressed with FK228, a potent HDAC (histone deacetylase) inhibitor, that eventually inactivates PAK1 (*2*), at least in cell culture (*4*), suggesting that some of ovarian cancers require the kinase PAK1 for their growth. However, none of these anti-PAK1 drugs are available on the market as yet. Moreover, Tam is known to be effective in suppressing the growth of only 10% of chemotherapeutics (DNA/MT poisons)-resistant ovarian cancers (*5*), suggesting that the remaining 90% of these ovarian cancers no longer depend on estrogen for their growth.

Thus, we recently identified a few anti-PAK1 products among those which are already available on the market. One of them is the CAPE (caffeic acid phenethyl ester)-based extract of NZ (New Zealand) propolis called Bio 30. CAPE and a few other anti-cancer ingredients in Bio 30 synergestically work to suppress very strongly the PAK1-dependent growth of breast and pancreatic cancers as well as NF (neurofibromatosis) tumors and glioma *in vivo* (xenografts in mice) (*6,7*). Another is Ivermectin, an old antibiotic which has been used as a potent anti-parasitic drug that kills mainly the intestinal worms (nematodes) by inhibiting their GABA receptor, but not mammalian counterparts (*8*). Recently we demonstrated that Ivermectin inactivates the kinase PAK1 in a variety of

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human ovarian cancer cell lines somehow, and blocks their growth (*9*), confirming that many ovarian cancers also require this kinase for their growth. However, their sensitivity to Ivermectin varies from one cancer cell line to another for an unknown reason, and the growth of normal cells is not inhibited by this drug at all (*9*). Interestingly, in many human ovarian cancer cells the PAK1 gene expression was found to be amplified along with a few other genes on 11q13 locus (*10,11*). In general, however, over-expression of PAK1 protein is a very rare event in human cancers, except for breast and ovarian cancers. In more than 70% of cancer cases, the kinase activity PAK1 is abnormally elevated instead, through the activation of upstream oncogenic signal transducers such as RAS, without any mutation on PAK1 gene itself.

In this study, using one of the direct PAK1 inhibitors called TAT-PAK18 which blocks the PAK1-PIX interaction essential for the PAK1 activation (*12,13*), we examined the possible relationship between the sensitivity of human ovarian cancer cell lines to TAT-PAK18 and PAK1 expression/its autophosphorylation (at Thr 423) levels, as TAT-PAK18 has been shown to block the autophosphorylation of PAK1 at Thr 423 (*14*), and inactivate this kinase both *in vitro* and *in vivo* (*13,14*).

2. Materials and Methods

2.1. *Reagents*

The peptide TAT-PAK18 (sequence: RKKRRQRRR-G-PPVIAPRPEHTKSVYTRS) was custom-synthesized by Calbiochem. This PAK1-inactivating peptide was synthesized by coupling the specific PAK1 kinase inhibitory domain of 18 amino acids (called PAK18), which blocks the PAK1-PIX interaction (*12,13*), to a cell-permeable highly basic peptide vector of 9 amino acids called TAT (*14,15*) through the linker Gly (G).

2.2. *Cell lines*

Human ovarian cancer cell lines TYK-nu, HTOA, SKOV3, RMUG-S and the control immortalized non-cancerous ovarian surface epithelial cell line HOSE were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), under the standard culture conditions.

2.3. *Immuno blot analysis*

For whole-cell lysates, samples were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL

leupeptin, and 1 mM PMSF). Protein samples were separated on 8% SDS-polyacrylamide gels (PAGE) and then transferred to polyvinylidene difluoride. Membranes were probed with antibodies to PAK1, phospho-PAK1 Thr 423, and p-Raf1 (Ser 338) (each diluted at 1:1,000, from Cell Signaling Technology, Danvers, MA, USA) as well as to β-actin (diluted at 1:500, from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After extensive washing with PBS containing 0.1% Tween 20, horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at dilutions of 1:5,000. Proteins were visualized using enhanced chemiluminescence and were normalized to β-actin.

2.4. *Cell viability (MTT) assay*

2×10^3 cells of each cancer cell line seeded per well were treated with varying concentrations of TAT-PAK18 (0.1-100 μM) for 72 h. Then cells were incubated at 37°C with 1 mg/mL MTT reagent (Sigma, St. Louis, MO, USA) for 3 h. Spectrophotometric absorbance of the samples was determined by the Ultra Multifunctional Microplate Reader (Tecan, Durham, NC, USA) at 595 nm.

2.5. *Assay for kinase activity of PAK1 (p-Raf1 levels)*

Since the Ser 338 of the kinase Raf1 is the major site to be phosphorylated by PAK1, the kinase activity of PAK1 in cells was routinely monitored by measuring the p-Raf1 level (phosphorylated at Ser 338) through the immuno-blot with the antibody against p-Raf1, which is far more sensitive and direct than measuring the p-PAK1 level (phosphorylated at Thr 423), after culturing the cancer cell lines such as RMUG-S in the presence of TAT-PAK18 (0.1-100 μM) or absence for 48 h as described previously (*9*).

3. Results and Discussion

3.1. *PAK1 expression levels and autophosphorylation at Thr 423 in human ovarian cancer cell lines compared with those in the control non-cancerous cell line*

Using the antibody against PAK1, we found that two cancer cell lines HTOA and SKOV3 express the PAK1 protein at a similar level to the non-cancerous cell line HOSE, while the cell line TYK-nu expresses at the significantly lower PAK1 level, and we could hardly detect the expression of PAK1 in the cancer cell line RMUG-S (see Figure 1, top row), although a much longer exposure of the immuno-blot revealed that RMUG-S cells indeed express PAK1, but only a very low level (data not shown). Interestingly, however, using the antibody against p-PAK1 (autophosphorylated at Thr 423), we could detect the p-PAK1 in only in the
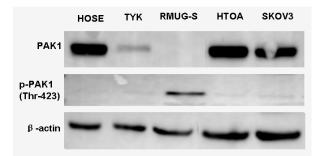
Figure 1. PAK1 protein/autophosphorylation levels in human ovarian cancer cell lines. PAK1 and p-PAK1 (autophosphorylated at Thr 423) levels in human ovarian cancer cell lines were compared with those in the control non-cancerous ovarian cell line (HOSE), using antibodies against PAK1 and p-PAK1, respectively, as described under "Materials and Methods". Only in RMUG-S cancer cells, PAK1 is autophosphorylated (abnormally activated).

Figure 2. Sensitivity of ovarian cancer cell lines to TAT-PAK18 in their growth. Four ovarian cancer cell lines were treated with TAT-PAK18 at the indicated concentrations for 72 h, and their growth was monitored by MTT methods as described under "Materials and Methods". Among these cell lines, RMUG-S, in which PAK1 is abnormally activated, was the most sensitive to TAT-PAK18 in their growth.

Figure 3. TAT-PAK18 down-regulates p-Raf1 level by inactivating PAK1 in RMUG-S cells. RMUG-S cells were treated with TAT-PAK18 at the indicated concentrations for 48 h, and the p-Raf1 (Ser 338) level was monitored by its antibody as described under "Materials and Methods".

cancer cell line RMUG-S, and none in the remaining cancer cell lines and the control non-cancerous cell line (see Figure 1, second row), clearly indicating that among these five human ovarian cell lines, only in RMUG-S cells, PAK1 is autophosphorylated at Thr 423 (abnormally activated), despite the fact that PAK1 (protein) is unexpectedly hypo-expressed in this cancer cell line.

3.2. *Sensitivity of ovarian cancer cell lines to TAT-PAK18 in their growth*

Although TAT-PAK18 inhibited the growth of all four cancer cell lines, RMUG-S was the most sensitive to this anti-PAK1 peptide (the IC_{50} was lower than 10 μM), and the IC_{50} of the remaining three cancer cell lines appears to be more than 10 times higher (above 100 μM) (see Figure 2).

3.3. *TAT-PAK18 inhibits the kinase activity of PAK1 in RMUG-S cells*

To prove that the effect of TAT-PAK18 on the growth of RMUG-S cell line is mainly due to its inactivation of the autophosphorylated PAK1, we have examined the effect of this peptide (0.1-100 μM) on the kinase activity of PAK1 in this cell line by monitoring the p-Raf1 (Ser 338) level. As shown in Figure 3, this direct PAK1 inhibitor clearly inactivates the PAK1 in RMUG-S cells around a range of its concentrations (1-10 μM) which strongly inhibit their growth.

These observations altogether suggest that the more PAK1 is abnormally activated, the more their growth is sensitive to TAT-PAK18. Since unlike FK228 and Ivermectin, TAT-PAK18 both selectively and directly inactivates PAK1, this finding provides the first direct evidence that ovarian cancers also require the kinase PAK1 for their growth. Interestingly, the sensitivity
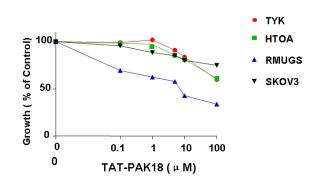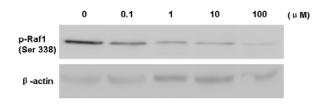
of these cancer cell lines to another anti-PAK1 drug Ivermectin (*9*) is rather opposite to their sensitivity to TAT-PAK18. The IC_{50} of TYK-nu was around 5 μM, while the IC_{50} of RMUG-S is around 20 μM, in both their growth and the kinase activity of PAK1 (monitored by p-Raf1 levels). This might be partly due to the possibility that like FK228, Ivermectin might inactivate another oncogenic kinase called AKT, in addition to the kinase PAK1, perhaps by blocking directly their upstream kinase(s) such as PI-3 kinase. For like FK228, Ivermectin can suppress even the growth of ovarian cancer cell line SKOV3 (*9*), which is highly resistant to another natural anti-PAK1 drug called menaquinone-7 (vitamin K_2) from a Japanese traditional cuisine called "Natto" (a fermented sticky soy bean product), which does not inactivate AKT (*16,17*), mainly due to the abnormally activated AKT in this cancer cell line (*18*). It is also possible that Ivermectin inactivates PAK1 not by blocking its auto-phosphorylation at Thr 423 (= the PAK1-PIX interaction), but by blocking other signaling pathways leading to the PAK1 activation such as Tyr-phosphorylation of PAK1 by another upstream kinase ETK (*3,19*).

We previously shown that a combination of two distinct anti-PAK1 drugs, AG 879/GL-2003 and PP1, which block the PAK1-ETK interaction and inactivate

Src family kinases, respectively, almost completely suppresses the PAK1-depepndent growth of human pancreatic and breast cancer xenografts in mice, due to their synergy (*3,13*). In this context, it would be of great interest for us to examine in the future whether Ivermectin and TAT-PAK18 or a few other PAK1 autophosphorylation inhibitors such as CEP-1347, OSU-03012 and Artepillin C (ARC) from Brazillian green propolis (*20-22*) also show such a synergy to suppress the PAK1-dependent growth of human ovarian cancer xenografts in mice.

References

1. Rayala S, Molli P, Kumar R. Nuclear p21-activated kinase 1 in breast cancer packs off tamoxifen sensitivity. Cancer Res. 2006; 66:5985-5988.
2. Hirokawa Y, Arnold M, Nakajima H, Zalcberg J, Maruta H. Signal therapy of breast cancers by the HDAC inhibitor FK228 that blocks the activation of PAK1 and abrogates the tamoxifen-resistance. Cancer Biol Ther. 2005; 4:956-960.
3. Hirokawa Y, Levitzki A, Lessene G, Baell J, Xiao Y, Zhu H, Maruta H. Signal therapy of human pancreatic cancer and NF1-deficient breast cancer xenograft in mice by a combination of PP1 and GL-2003, anti-PAK1 drugs (Tyr-kinase inhibitors). Cancer Lett. 2007; 245:242-251.
4. Khabele D, Son DS, Parl AK, Goldberg GL, Augenlicht LH, Mariadason JM, Rice VM. Drug-induced inactivation or gene silencing of class I histone deacetylases suppresses ovarian cancer cell growth: implications for therapy. Cancer Biol Ther. 2007; 6:795-801.
5. Tropé C, Marth C, Kaern J. Tamoxifen in the treatment of recurrent ovarian carcinoma. Eur J Cancer. 2000; 36 (Suppl 4):S59-S61.
6. Demestre M, Messerli SM, Celli N, Shahhossini M, Kluwe L, Mautner V, Maruta H. CAPE (caffeic acid phenethyl ester)-based propolis extract (Bio 30) suppresses the growth of human neurofibromatosis (NF) tumor xenografts in mice. Phytother Res. 2009; 23:226-230.
7. Messerli S, Li XN, Maruta H. Bio 30, the anti-PAK1 extract of NZ (New Zealand) propolis, blocks the growth of glioma from both adults and children grafted in mice. Submitted, 2010.
8. Wang CC, Pong SS. Actions of avermectin B1a on GABA nerves. Prog Clin Biol Res. 1982; 97:373-395.
9. Hashimoto H, Messerli SM, Sudo T, Maruta H. Ivermectin inactivates the kinase PAK1 and block the PAK1-dependent growth of human ovarian cancer and NF2 tumor cell lines. Drug Discov Ther. 2009; 3:243-246.
10. Xie D, Yang GF, Chen YQ, Jiang LF, Xiao LZ. Significance and mechanisms of overexpression of PAK1 gene in epithelial ovarian neoplasms. Zhonghua Zhong Liu Za Zhi. 2006; 28:911-914. (in Chinese)
11. Brown LA, Kalloger SE, Miller MA, Shih IeM, McKinney SE, Santos JL, Swenerton K, Spellman PT, Gray J, Gilks CB, Huntsman DG. Amplification of 11q13 in ovarian carcinoma. Genes Chromosomes Cancer. 2008; 47:481-489.
12. Obermeier A, Ahmed S, Manser E, Yen SC, Hall C, Lim L. PAK promotes morphological changes by acting upstream of Rac. EMBO J. 1998; 17:4328-4339.
13. He H, Hirokawa Y, Manser E, Lim L, Levitzki A, Maruta H. Signal therapy for RAS-induced cancers in combination of AG 879 and PP1, specific inhibitors for ErbB2 and Src family kinases, that block PAK activation. Cancer J. 2001; 7:191-202.
14. Zhao L, Ma QL, Calon F, Harris-White ME, Yang F, Lim GP, Morihara T, Ubeda OJ, Ambegaokar S, Hansen JE, Weisbart RH, Teter B, Frautschy SA, Cole GM. Role of PAK pathway defects in the cognitive deficits of Alzheimer disease. Nat Neurosci. 2006; 9:234-242.
15. Kiosses WB, Hood J, Yang S, Gerritsen ME, Cheresh DA, Alderson N, Schwartz MA. A dominant-negative p65 PAK peptide inhibits angiogenesis. Circ Res. 2002; 90:697-702.
16. Liu W, Nakamura H, Yamamoto T, Ikeda N, Saito M, Ohno M, Hara N, Imanishi H, Shimomura S, Yamamoto T, Sakai T, Nishiguchi S, Hada T. Vitamin K_2 inhibits the proliferation of $HepG_2$ cells by up-regulating the transcription of p21 gene. Hepatol Res. 2007; 37:360-365.
17. Shibayama-Imazu T, Sakairi S, Watanabe A, Aiuchi T, Nakajo S, Nakaya K. Vitamin K_2 selectively induced apoptosis in ovarian TYK-nu and pancreatic MIA PaCa-2 cells out of eight solid tumor cell lines through a mechanism different from geranylgeraniol. J Cancer Res Clin Oncol. 2003; 129:1-11.
18. Matei D, Chang DD, Jeng MH. Imatinib mesylate (Gleevec) inhibits ovarian cancer cell growth through a mechanism dependent on platelet-derived growth factor receptor alpha and Akt inactivation. Clin Cancer Res. 2004; 10:681-690.
19. He H, Hirokawa Y, Gazit A, Yamashita Y, Mano H, Kawakami Y, Kawakami, Hsieh CY, Kung HJ, Lessene G, Baell J, Levitzki A, Maruta H. The Tyr-kinase inhibitor AG879, that blocks the ETK-PAK1 interaction, suppresses the RAS-induced PAK1 activation and malignant transformation. Cancer Biol Ther. 2004; 3:96-101.
20. Nheu TV, He H, Hirokawa Y, Tamaki K, Florin L, Schmitz ML, Suzuki-Takahashi I, Jorissen RN, Burgess AW, Nishimura S, Wood J, Maruta H. The K252a derivatives, inhibitors for the PAK/MLK-kinase family, selectively block the growth of RAS transformants. Cancer J. 2002; 8:328-336.
21. Porchia LM, Guerra M, Wang YC, Zhang Y, Espinosa AV, Shinohara M, Kulp SK, Kirschner LS, Saji M, Chen CS, Ringel MD. OSU-03012, a celecoxib derivative, directly targets PAK. Mol Pharmacol. 2007; 72:1124-1131.
22. Messerli SM, Ahn MR, Kunimasa K, Yanagihara M, Tatefuji T, Hashimoto K, Mautner V, Uto Y, Hori H, Kumazawa S, Kaji K, Ohta T, Maruta H. Artepillin C (ARC) in Brazilian green propolis selectively blocks the oncogenic PAK1 signaling and suppresses the growth of NF tumors in mice. Phytother Res. 2009; 23:423-427.